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# Molecular characterization of Iranian wheat stripe virus shows its taxonomic position as a distinct species in the genus *Tenuivirus**

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**Summary.** The full lengths of three genome segments of Iranian wheat stripe virus (IWSV) were amplified by reverse transcription (RT) followed by polymerase chain reaction (PCR) using a primer complementary to tenuivirus conserved terminal sequences. The segments were sequenced and found to comprise 3469, 2337, and 1831 nt, respectively. The gene organization of these segments is similar to that of other known tenuiviruses, each displaying an ambisense coding strategy. IWSV segments, however, are different from those of other viruses with respect to the number of nucleotides and deduced amino acid sequence for each ORF. Depending on the segment, the first 16–22 nt at the 5′ end and the first 16 nt at the 3′ end are highly conserved among IWSV and rice hoja blanca virus (RHBV), rice stripe virus (RSV) and maize stripe virus (MStV). In addition, the first 15–18 nt at the 5′ end are complementary to the first 16–18 nt at the 3′ end. Phylogenetic analyses showed close similarity and a common ancestor for IWSV, RHBV, and Echinochloa hoja blanca virus (EHBV). These findings confirm the position of IWSV as a distinct species in the genus *Tenuivirus*.

*Nucleotide sequence data reported are available in the GenBank databases under the accession numbers AY 312434, AY 312435 and AY 312436 for IWSV RNA2, 3 and 4, respectively.

## Introduction

The genus *Tenuivirus* comprises a number of unusual plant viruses that cause significant diseases in economically important crop plants including rice and maize [8]. Definitive species within this genus are *Rice stripe virus* (RSV) (type), *Maize stripe virus* (MStV), *Rice hoja blanca virus* (RHBV), *Rice grassy stunt virus* (RGSV), and *Echinochloa hoja blanca virus* (EHBV). Six lesser-known viruses, European wheat striate mosaic virus (EWSMV), Iranian wheat stripe virus (IWSV), Brazilian wheat spike virus, rice wilted stunt virus, winter wheat mosaic virus, and *Urochloa* hoja blanca virus are tentatively included in this genus [25]. IWSV was first detected in wheat fed on by its vector, *Unkanodes tanasijevici*, and was reported in southern Iran in 1989 [11]. This virus has many characteristics in common with members of the *Tenuivirus* genus including particle morphology, host range, vector type, production of non-structural protein (NS) in host tissue, and a multicomponent nature of the genome. In addition, IWSV is serologically related to RHBV [12].

Until now there has been no information regarding genome structure, organization, and expression strategy of IWSV. The aim of the present study was to characterize the genome and obtain further evidence regarding the taxonomic position of this virus.

## Materials and methods

### *Sources of IWSV*

IWSV-infected wheat was collected in wheat fields in Badjgah (15 km north of Shiraz, Iran). Infection of collected plants was confirmed by testing them against IWSV antiserum in an agar gel diffusion test [12]. Before experimentation, IWSV-infected wheat was checked serologically to assure it was free of other planthopper-borne viruses of cereals found in Iran, namely barley yellow striate mosaic virus, Iranian maize mosaic virus, and maize rough dwarf virus.

The virus was propagated in a greenhouse by inoculation of wheat plants using *U. tanasijevici*. One viruliferous planthopper was used per plant at one leaf stage. Leaf tissues were harvested once symptoms of infection were apparent and were used freshly for experimentation or stored at −20 °C until required. IWSV was purified according to Heydarnejad and Izadpanah [12].

### *Extraction of virus RNA*

Fifty μl of virus suspension (containing approximately 1 mg nucleoprotein) was mixed with an equal volume of phenol:chloroform:iso-amyl alcohol (25:24:1) containing 0.1% (w/v) 8-hydroxyquinoline to form an emulsion. After centrifuging for 2 min at 14,000 rpm in a benchtop microfuge, the aqueous phase containing the RNA was collected. The extraction was repeated two times followed by two extractions using chloroform:iso-amyl alcohol (24:1). The final aqueous phase was mixed with 3 volumes of absolute ethanol and 1/10 volume of 2.5 M potassium acetate, pH 5.2. The mixture was stored for at least 30 min at −20 °C before centrifuging at 14,000 rpm for 10 min in a benchtop centrifuge. The pellet was washed twice with 70% ethanol and finally resuspended in 100 μl DEPC-treated water. The RNA was stored at −20 °C.

*Electrophoresis*

Analysis of IWSV-RNA and PCR products was performed by non-denaturing electrophoresis in 1% agarose gel containing 1× Tris-borate/EDTA (TBE buffer, pH 8.0) or 1× Tris-acetate/EDTA (TAE buffer, pH 8) and 0.5 μg/ml (w/v) ethidium bromide. Size markers of 1 kb DNA Ladder (Gibco BRL) and 0.24–9.5 Kb RNA Ladder (Gibco BRL) were used.

*cDNA synthesis*

Three μg of IWSV-RNA extracted from the ribonucleoprotein (RNP) were converted to cDNA using 20 pmol of the synthetic oligonuleotide 5′ AAGGCCTTGCGGCCGCACACAA AGTC 3′ (JHG1) as primer. The 3′ terminal 10 bases of the primer are complementary to the highly conserved termini of tenuivirus segments 2, 3, and 4. The bases 9–16 in the primer contained a *Not* I restriction site. Reverse transcription was carried out with AMV reverse transcriptase (RT) (Promega) in the presence of RNase inhibitor (Pharmacia) for 45 min at 42 °C. RT was destroyed by heating at 95 °C for 5 min.

*Amplification of cDNA by polymerase chain reaction (PCR)*

cDNA was amplified by PCR using 100 pmol of JHG1 primer and 2 units of *Vent* DNA polymerase (New England Biolabs). The PCR thermal profile was 30 cycles of 94 °C for 50 s, 60 °C for 60 s, and 72 °C for 60 s, followed by one cycle of 72 °C for 300 s. Amplified segments were analyzed by agarose gel electrophoresis. The PCR products were extended by *Taq* polymerase for 10 min at 72 °C to allow addition of an A to the 3′ end and their subsequent insertion into the TOPO TA cloning vector pCR 2.1 (Invitrogen). Purified recombinant plasmids were digested by *Not* I restriction enzyme (BioLabs Inc.) to screen for the presence of insert.

*Sequencing*

Recombinant plasmids were sequenced in both directions by extending M13 reverse and forward primers, using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit and ALF-express DNA sequencer (Amersham-Pharmacia Biotech, Ltd.). The sequence data were analyzed using the DNA-Star analysis software package and compared with known tenuivirus segments. Nucleotide and deduced amino acid sequence homology between different RNA segments and encoded proteins were calculated using the DNAMAN software package (Lynnon Biosoft, Quebec, Canada).

*Phylogenetic analyses*

Phylogenetic analyses were performed using PAUP*, Version 4.0 b8 [21]. Three data sets were used separately in order to analyze the phylogenetic relationship of IWSV with different members of *Tenuivirus* and some members of *Phlebovirus* genera. The first data group contained either amino acid or related RNA sequences of the nucleocapsid (N) protein. The second group consisted of either amino acid or related RNA sequences of virion membrane glycoprotein (membrane glycoprotein-like; pc2 protein). The third group consisted of a combined matrix of either amino acid or RNA sequence of both proteins or related genes. Oropouche virus (OPV) from the *Bunyavirus* genus or TSWV from the *Tospovirus* genus was used as an outgroup. Both of these viruses belong to the *Bunyaviridae* family [7, 20], and a database search using their membrane glycoproteins and N proteins showed no similarity with the counterpart proteins of IWSV. Due to a lack of sequence information for the virion membrane glycoprotein of EHBV, only N protein and related RNA sequences of this virus were used in phylogenetic studies. Each data set, *i.e.* amino acid or related RNA sequences, of

both proteins were aligned separately using CLUSTAL, and regions of ambiguous alignment or data absence were excluded. Cladistic analyses were performed using maximum parsimony, and standard settings were used for the searching option of PAUP*. A phylogenic tree was constructed by a heuristic search for each data set. The analysis was replicated 1000 times. Bootstrapping was performed to estimate the stability and support for the extracted tree.

## Results

### *Composition of the IWSV genome*

The composition of the IWSV genome was investigated by separating purified RNAs by gel electrophoresis. Seven bands of IWSV RNA were visualized in a non-denaturing gel. The sizes of the four smaller bands were estimated to be 1.8, 2.2, 3.2, and 6.9 kb (Fig. 1).

cDNA synthesis and PCR were used to generate plasmids containing IWSV sequences. The 3′ terminal 10 bases of the JHG1 primer are complementary to the highly conserved termini of tenuivirus segments 2, 3, and 4. This primer was used to reverse transcribe and then to amplify cDNAs from tenuivirus RNAs. Five to seven bands were obtained following electrophoresis of PCR products through a 1% agarose gel (Fig. 2). Based on the size marker, the estimated number of

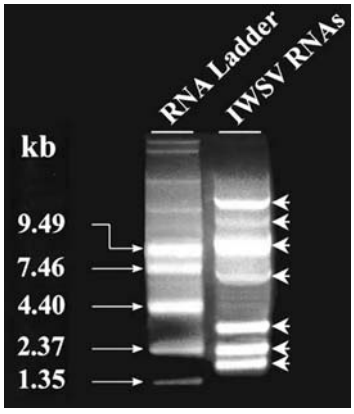


**Fig. 1.** Band pattern of IWSV RNA in non-denaturing 1% agarose gel

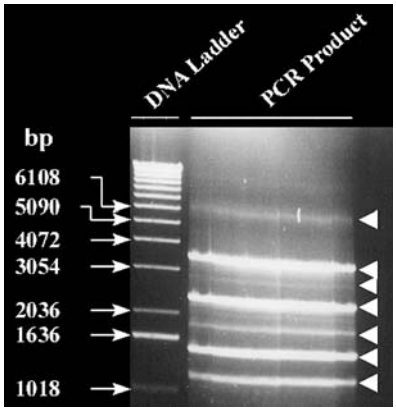


**Fig. 2.** RT-PCR analysis of IWSV RNA segments. cDNAs were amplified by PCR using *Vent* polymerase and JHG1 primer

nucleotides for these bands were: 1.1, 1.4, 1.8, 2.3, 3.5, and 5.7 kbp. Some of these values (1.8, 2.3, and 3.5 kb) approximate those for RNA bands in Fig. 1. By analogy with other tenuiviruses and on the basis of sequencing data (see below) these segments were designated 4, 3, and 2, respectively.

IWSV PCR products were cloned into the pCR 2.1-TOPO vector. Since the PCR primer JHG1 contained a *Not* I restriction enzyme site at its terminus, digestion of the recombinant plasmids with *Not* I revealed the insert sizes of tenuivirus-specific cloned products. No *Not* I site was detected within full-length amplified IWSV segments 2, 3, and 4. A variety of insert sizes were obtained (data not shown). Subsequently, nucleotide sequencing showed that some clones contained cDNA for full-length IWSV segments 2, 3, and 4, while the rest of the screened clones (e.g., the two smallest bands in Fig. 2) contained incomplete lengths of these segments. Addition of a poly A tail to the 3′ end of IWSV RNAs in order to sequence the 5′ and 3′ ends was not successful.

### *IWSV segment 2*

IWSV RNA segment 2 comprises 3469 nucleotides with a base composition of 30.2% A, 17.6% G, 20.2% C, and 31.9% U. This segment is ambisense, having the capacity to encode two proteins (Fig. 3). The viral sense ORF near the 5′ end extends from nt 83 to nt 691. The deduced translation product contains 202 amino acids with $M_r$ of 27000. The complementary ORF extends from nt 69 to nt 2573 of vcRNA and potentially encodes a protein of 834 amino acids with $M_r$ of 109260 (Table 1). The intergenic region between these two ORFs contains 205 nt, and is rich in oligo (U) sequences. The first 19 nt at the 5′ end and the first 16 nt

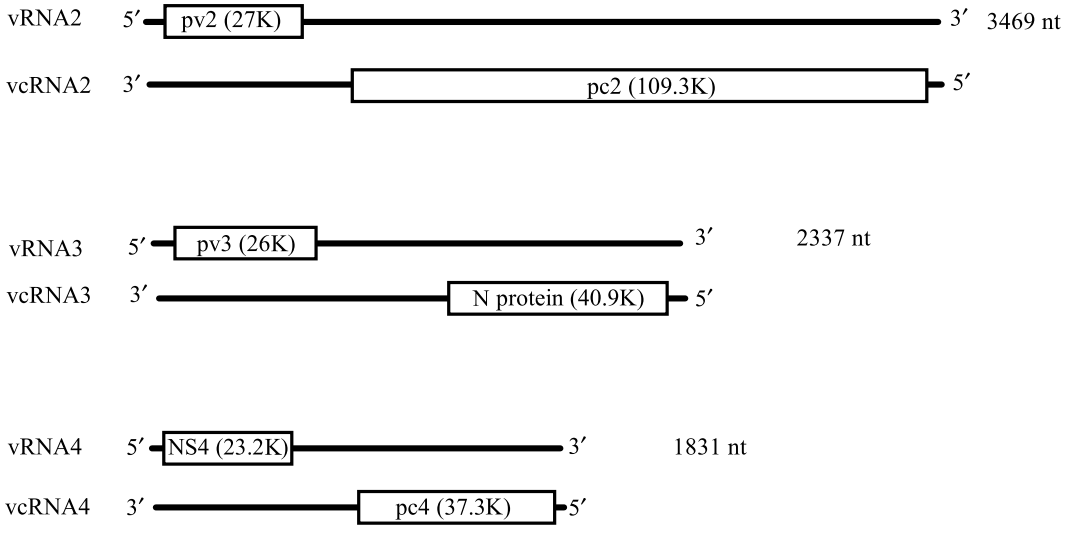


**Fig. 3.** Schematic representation of ORFs on the v and vc sequences of IWSV RNA2, RNA3, and RNA4. RNA segments are displayed as black bars. Putative proteins are shown as boxes each with its predicted molecular mass (kDa). See Table 1 for abbreviations

**Table 1.** Coding capacity and characteristics of the deduced products of IWSV genome segments 2–4 using sequencing data

| Segment | Number of nucleotides | Intergenic region | RNA sense | 5′ Non coding region | Number of nucleotides for each ORF | Number amino acids | Molecular mass of predicted ORF product | Counterpart proteins in other tenuiviruses |
|---|---|---|---|---|---|---|---|---|
| RNA 2 | 3469 | 205 | vRNA 2 | 82 | 609 | 202 | 27,003 | pv2[a] |
| | | | vcRNA 2 | 68 | 2505 | 834 | 109,258 | pc2[a] |
| RNA 3 | 2337 | 602 | vRNA 3 | 72 | 594 | 197 | 25,970 | NS3[b] |
| | | | vcRNA 3 | 115 | 954 | 317 | 40,908 | N[b] |
| RNA 4 | 1831 | 361 | vRNA 4 | 53 | 525 | 174 | 23,106 | NS4 or NCP[c] |
| | | | vcRNA 4 | 37 | 855 | 284 | 37,275 | pc4[c] |

Abbreviations: N, nucleocapsid protein; NCP, non-capsid protein; NS, non-structural protein; ORF, open reading frame; pc, proteins that are encoded by virus complementary RNAs; pv, proteins that are encoded by viral RNAs; vRNA, virus RNA and vcRNA, virus complementary RNA

Information for counterpart proteins in other tenuiviruses derived from:

[a][2, 5, 6, 22, 24]

[b][15, 1, 3, 18, 24, 26]

[c][4, 14, 16, 17, 19, 27]

at the 3′ end are highly conserved between the RNAs 2 of RHBV, RSV, MStV, and IWSV [2, 5, 6, 22, 24], bearing in mind the caveat that the extreme termini of IWSV sequence had been predetermined by use of primer complementary to the first 10 nucleotides at each end of the genome. The terminal complementarity of the genome extends for 17 nucleotides, with a single nucleotide in the 5′ terminus being unpaired.

### *IWSV segment 3*

The length of IWSV RNA3 is 2337 nt (Fig. 3) and has the base composition: A, 28.1%; G, 17.7%; C, 22.5% and U, 31.7%. This segment has two ORFs in an ambisense arrangement. The ORF in the viral sense RNA begins at nucleotide position 73 and continues to a UGA stop codon at the position 664–666. The predicted translation product contains 197 amino acids with a molecular mass of 26 kDa. The other ORF, spanning nt 116–1066 from the 5′ end of the complementary sense RNA, had the capacity to encode a protein of 317 amino acids with a molecular mass of 41 kDa (Table 1). Between the two ORFs, there is a U-rich non-coding intergenic region consisting of 606 nt. In addition, an 88 nt section in the intergenic region (nt 824–912) is highly homologous to the corresponding sequences in RHBV and EHBV. The first 22 nt at the 5′ end and the first 16 nt at the 3′ end of the segment are also highly conserved among RNA3s of RHBV, RSV, MStV, and IWSV [1, 3, 15, 18, 24, 26]. As for RNA2, terminal complementarity extends beyond the absolutely conserved termini; in this case 16 of the first 17 nt at the 5′ end are perfectly complementary to the first 16 nt at the 3′ end.

### *IWSV segment 4*

IWSV RNA4 contains 1831 nt with a base composition of 30.6% A, 19.1% G, 21.7% C, and 28.5% U and has an ambisense coding strategy (Fig. 3). A 525 nt ORF at the 5′ end of viral sense RNA begins at nucleotides 54–56. This p4 protein has 174 amino acids and a predicted molecular mass of 23.2 kDa. The other ORF on the complementary strand of RNA comprises 852 nt (nt 38–892). The predicted protein contains 284 amino acids with a molecular mass of 37.3 kDa. Within the long intergenic non-coding region (nt 579–939) there is a 22 nt section which is conserved among the RNA4s of RHBV, RSV, MStV, EHBV, and IWSV. In addition, the first 16 nt at the 5′ and 3′ termini are highly conserved between these viruses [4, 16, 17, 19, 24, 27]. Again, there is perfect complementarity between the 5′ and 3′ termini.

### *Phylogenetic analyses*

Maximum parsimony analyses using amino acid and RNA sequences of the N protein and virion membrane glycoprotein (pv2) as well as combined matrix data were undertaken. The topology of all constructed trees was similar with respect to the position of IWSV among members of *Tenuivirus* genus (some phylogeny trees not presented). All analyses indicated very strong support

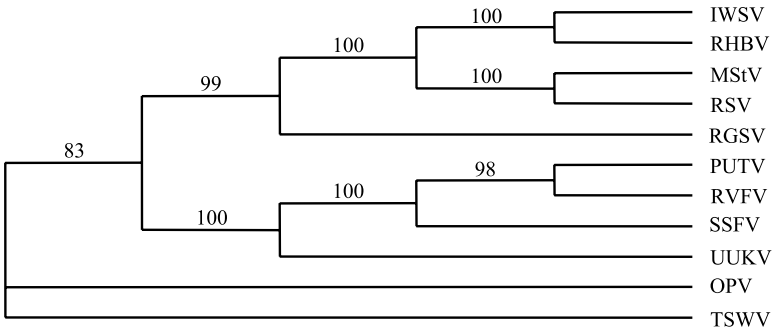


**Fig. 4.** The most parsimonious tree of IWSV, tenuiviruses (RSV, MStV, RHBV, and RGSV), phleboviruses (Punta Toro virus, PUTV, Rift Valley fever virus, RVFV, Sicilian sandfly fever virus, SSFV and Uukuniemi virus, UUKV) and Oropouche virus, OPV. TSWV was used as an outgroup virus. The phylogenetic tree was produced using a combined matrix of virion membrane glycoprotein (membrane glycoprotein-like or pc2 protein) and nucleocapsid (N) protein. Bootstrap values indicate the percentage support for individual branches based on 1000 replicates. Accession numbers for sequence data are as follows: RSV, D13176; MStV, U53224; RHBV, L54073; RGSV, AB010376; UUKV, M17417; RVFV, M11157; PUTV, M1156; TSWV, S48091; SFSV, U30500, and OPV, AF312381

(*i.e.* bootstrap values between 98–100%) for IWSV and other tenuiviruses (with the exception of RGSV) being a monophyletic group. There was also strong support (*i.e.* bootstrap values between 90–99%) of an early divergence of RGSV from other tenuiviruses when combined matrix data or amino acid and related RNA sequences of the pv2 protein were analyzed. However, analysis of amino acid or RNA sequences of the N protein only moderately supported this divergence (*i.e.* bootstrap values between 72–75%). A constructed tree using a combined matrix of virion membrane glycoprotein (membrane glycoprotein-like or pc2 protein) and nucleocapsid (N) protein showed a monophyletic topology or a common ancestor for both *Tenuivirus* and *Phlebovirus* genera with 83% bootstrap support (Fig. 4).

## Discussion

Since the RNAs that make up the genomes of tenuiviruses can exist in single stranded and double stranded forms, gel patterns of RNA segments can give rise to several different species with different migration characteristics. cDNA synthesis and subsequent PCR amplification of the product using JHG1 primer were successfully carried out. The exact numbers of nucleotides in each of the segments 2, 3, and 4 are reported.

Sequence analysis of the IWSV genome revealed that segments 2, 3, and 4 comprise 3469, 2337, and 1831 nt, respectively, and these numbers are close to the lengths of the corresponding segments in other tenuiviruses, especially RHBV and EHBV. As for other tenuivirus family members, each IWSV RNA segment studied contains two ORFs arranged in an ambisense orientation. The sizes of

IWSV proteins are slightly larger than the corresponding proteins in the other tenuiviruses [23, 24, reviewed in 10].

The high level of homology, at both the nucleotide and amino acid levels, between corresponding tenuivirus genes suggests that their functions are similar (Fig. 5). Characteristics of genome segments and their putative products are shown in Table 1. Deduced amino acid sequence homology between different proteins encoded by six IWSV ORFs on three segments and their counterparts in other tenuiviruses (excluding RGSV) is between 41.5–81.6%. In addition, nucleotide homology between IWSV intergenic regions for three RNA segments and their counterparts in other tenuiviruses (excluding RGSV) was found to be between 34.4–58.8.

a) 27K protein (vRNA2)

| | | | | |
|---|---|---|---|---|
| | | | | **IWSV** |
| | | | **RSV** | 42.7 % |
| | | **MStV** | 60.8 % | 44.8 % |
| | **RHBV** | 46.7 % | 39.4 % | 68.5 % |
| **RGSV** | 22.9 % | 22.3 % | 19.4 % | 22.3 % |

b) 109.3 K protein (vcRNA2)

| | | | | |
|---|---|---|---|---|
| **IWSV** | | | | |
| 42.4 % | **RSV** | | | |
| 41.5 % | 53.0 % | **MStV** | | |
| 69.1 % | 42.9 % | 41.9 % | **RHBV** | |
| 19.5 % | 16.6 % | 16.7 % | 17.4 % | **RGSV** |

c) 26K protein (vRNA3)

| | | | | |
|---|---|---|---|---|
| | | | | **IWSV** |
| | | | **RSV** | 46.2 % |
| | | **MStV** | 64.0 % | 49.2 % |
| | **RHBV** | 48.2 % | 45.8 % | 79.2 % |
| **EHBV** | 96.5 % | 48.7 % | 46.7 % | 79.2 % |
| **RGSV*** | 15.8 % | 16.4 % | 16.9 % | 15.3 % | 16.9 % |

d) 40.93 K protein (vcRNA3)

| | | | | | |
|---|---|---|---|---|---|
| **IWSV** | | | | | |
| 46.3 % | **RSV** | | | | |
| 42.7 % | 65.2 % | **MStV** | | | |
| 69.9 % | 47.0 % | 46.2 % | **RHBV** | | |
| 69.4 % | 46.3 % | 45.5 % | 89.9 % | **EHBV** | |
| 17.8 % | 15.1 % | 14.8 % | 19.0 % | 18.4 % | **RGSV*** |

e) 23.1K protein (vRNA4)

| | | | | |
|---|---|---|---|---|
| | | | | **IWSV** |
| | | | **RSV** | 60.9 % |
| | | **MStV** | 73.7 % | 59.5 % |
| | **RHBV** | 59.0 % | 58.0 % | 81.6% |
| **EHBV** | 93.7 % | 57.2 % | 57.5 % | 81.6 % |
| **RGSV*** | 27.2 % | 26.6 % | 25.0 % | 24.6 % | 26.6 % |

f) 37.3K protein (vcRNA4)

| | | | | | |
|---|---|---|---|---|---|
| **IWSV** | | | | | |
| 60.6 % | **RSV** | | | | |
| 58.0 % | 76.3 % | **MStV** | | | |
| 76.7 % | 57.2 % | 56.5 % | **RHBV** | | |
| 79.5 % | 57.6 % | 55.5 % | 88.7 % | **EHBV** | |
| 24.3 % | 21.9 % | 22.5 % | 24.7 % | 26.9 % | **RGSV*** |

**Fig. 5.** Comparison of putative amino acid sequence homology between different proteins encoded by Iranian wheat stripe virus (IWSV) ORFs and their counterparts in other tenuiviruses. **a**, protein encoded by vRNA2; **b**, protein encoded by vcRNA2; **c**, protein encoded by vRNA3; **d**, protein encoded by vcRNA3; **e**, protein encoded by vRNA4; **f**, protein encoded by vcRNA4. Abbreviations are: vRNA, virus RNA; vcRNA, virus complementary RNA. *RGSV RNA5 and RNA6 correspond to RNA3 and RNA4 of other tenuiviruses, respectively

Phylogenetic analyses using either amino acid and related RNA sequence of nucleocapsid (N) protein or virion membrane glycoprotein (membrane glycoprotein-like or pv2), confirmed a close similarity and suggested a common ancestor for IWSV, RHBV, and EHBV with a strong bootstrap support of 100%. Furthermore, it has been demonstrated that IWSV is serologically related to RHBV [12, 13] when RHBV antisera to coat protein and major non-structural protein are used. On the other hand, antiserum against major non-structural protein of EHBV did not react with IWSV-infected wheat sap [13]. Thus, although there are close similarities in the sequences of IWSV and EHBV (Fig. 5), these two viruses are clearly serologically distinct from each other.

The difference between EHBV and IWSV is also reflected in their host ranges. The grass *Echinochloa colona* is the main host for EHBV, but all attempts to infect this grass and other related species (*E. cruss-galli*) by IWSV under experimental conditions, failed (data not presented). This observation and the close similarities between RHBV and IWSV support the hypothesis that rice may have been a natural host of IWSV in Iran.

In conclusion, evidence based on phylogeny, serology, and biological characteristics [12, 13] supports the inclusion of IWSV as a definitive member of the *Tenuivirus* genus and places it at a close position to, but distinct from, EHBV and RHBV.

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